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(57) Abstract

DNA comprising tissue specific regulatory regions are disclosed. The DNA is useful in conjunction with other gene sequences for introduction into plant cells to provide transformed plants having tissues with a modified phenotypic property. The invention is exemplified with tomato fruit specific promoters which are active at climacteric and throughout the stages of fruit ripening.

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TISSUE-SPECIFIC AND DEVELOPMENTALLY REGULATED TRANSCRIPTIONAL SEQUENCES AND USES THEREOF

FIELD OF THE INVENTION

This invention relates generally to plant biotechnology and specifically to DNA sequences capable of directing tissue-specific and developmentally regulated expression of gene fusion constructs in transgenic plants.

INTRODUCTION

Development of new crop plants through traditional plant breeding methods relies upon the observation of plant characteristics (phenotypes) and studies of their inheritance. Plant breeders have identified numerous desirable phenotypes and, through controlled breeding efforts, transferred these phenotypes into commercial plant varieties. However, in the process of transferring desirable phenotypes, undesirable phenotypes also can be transferred. Breeders must then perform numerous procedures which eventually remove all but the desired phenotype. As a result, the effort required to transfer a single trait may take from five to twenty-five years. This major limitation of conventional breeding techniques can be overcome by applying the techniques of molecular biology and plant tissue culture.

Plants are highly evolved multicellular organisms. The hereditary material of plants, deoxyribonucleic acid or DNA, is contained within chromosomes which are comprised of genes encoding proteins. The specificity of expression of each gene is controlled by a regulatory region (*i.e.*, a transcriptional initiating sequence or promoter) associated with it. The gene is transcribed into ribonucleic acid (RNA) which is then translated into protein. Proteins are key molecules in the plant cell, comprising enzymes which control biochemical events and structural molecules which provide a framework for cell components.

The production of transgenic plants begins with the introduction of new genetic material into a single plant cell. The next step, the production

of a whole, transgenic plant, is greatly facilitated by the fact that plants, unlike most animals, can be regenerated asexually from such a single cell or a small piece of tissue.

Genetic engineering of plants is accomplished by isolating and characterizing genes of interest, splicing them to desirable promoters, and transferring them to plant cells or tissues which are then regenerated to produce transgenic plants. As a result of this process, the transgenic plants contain the transferred genetic information in their chromosomes. This genetic information is inherited in subsequent generations and confers a new phenotype upon the progeny plants.

It is frequently desirable for the promoters used in the production of transgenic plants to be capable of conferring specificity of expression upon the transgenic construct. One aspect of this desirability is the ability to manipulate phenotypes in fruit in order to produce fruit which will have improved characteristics such as solids content, flavor, texture, processing qualities, and the like.

It is an object of this invention to provide transcriptional sequences that are useful in the production of transgenic plants, and are also capable of conferring specificity of expression upon the transgenic construct. It is another object of the invention to provide transcriptional sequences that can be used to manipulate phenotypes in fruit in order to produce fruit which will have improved characteristics such as solids content, flavor, texture, processing qualities, and the like.

SUMMARY OF THE INVENTION

Novel DNA transcriptional sequences are provided which are capable of conferring upon gene fusion constructs the characteristics of tissue specific and developmentally regulated expression. In particular, DNA transcriptional sequences are provided which cause expression to occur in a tissue-specific manner, *e.g.*, a fruit-specific manner, at specific times during development, e.g., during fruit ripening. The transcriptional sequences are exemplified by sequences from clone λUC82-3.3, which is disclosed and claimed herein as **SEQ ID NO. 2**. The invention also provides sequences from

clone pTOMUC82.1, which is disclosed and claimed herein as **SEQ ID NO. 1.** Clone PTOMUC82.1 encodes a histidine decarboxylase-like protein (HDC-like). Sequences from **SEQ ID NO. 1**, are useful, for example, as probes for identifying and isolating genes that may have tissue-specific, developmentally regulatable promoters. Once identified, these promoter regions can be isolated. The invention provides gene fusion constructs containing the novel DNA sequences of **SEQ ID NO. 2**, thus enabling the production of high levels of RNA and, as appropriate, polypeptides (*e.g.,* reporter proteins, enzymes, *etc.*) in specific tissues, and at specific times during development, for example, during formation and ripening of fruit. The invention also provides transgenic plants and plant materials which contain gene fusion constructs containing the novel sequences of the invention operatively linked to at least one structural gene.

DESCRIPTION OF THE DRAWING

Figure 1 shows restriction enzyme maps illustrating the derivation of the pUC82-3.3SB (S=*Sst*I, B=*Bgl*II) insert from the insert of λUC82-3.3 (**SEQ ID NO. 2**). At the bottom of the figure is a schematic diagram of the nucleotide sequence of PUC82-3.3SB, with exons indicated by filled boxes. The percent sequence similarity between this genomic sequence and the HDC-like coding sequence (PTOMUC82.1; **SEQ ID NO. 1**) is indicated below each exon.

DEFINITIONS

In the present specification and claims, reference will be made to phrases and terms of art which are expressly defined for use herein as follows:

As used herein, promoter refers to a non-coding region of DNA involved in binding of RNA polymerase and other factors that initiate or modulate transcription whereby an RNA transcript is produced. Promoters can be naturally occurring or synthetically produced. Promoters, depending upon the nature of the regulation, may be constitutive or regulated. A constitutive promoter is always turned on. A regulatable promoter requires specific signals in order for it to be turned on or off. A developmentally regulated promoter is one that is turned on or off as a function of

development. A tissue-specific promoter is one that is turned on or off as a function of the tissue in which it is present.

In the present specification and claims, the terms promoter, transcriptional sequences, transcriptional initiating sequences and gene regulatory region are used interchangeably.

As used herein, the terms operatively linked, functionally linked or associated, or grammatical variations thereof, are equivalent terms that are used interchangeably. In particular these terms refer to the linkage of a promoter or a non-coding gene regulatory sequence to an RNA-encoding DNA sequence, and especially to the ability of the regulatory sequence or promoter to induce production of RNA transcripts corresponding to the DNA-encoding sequence when the promoter or regulatory sequence is recognized by a suitable polymerase. All three terms mean that linked DNA sequences (*e.g.*, promoter(s), structural gene (*e.g.*, reporter gene(s)), terminator sequence(s), *etc.*) are operational or functional, *i.e.*, work for their intended purposes. Stated another way, operatively or functionally linked, or associated, means that after the respective DNA segments are joined, upon appropriate activation of the promoter, the structural gene will be expressed.

As used herein, suitable plant material means and expressly includes, plant protoplasts, plant cells, plant callus, plant tissues, developing plantlets, immature whole plants and mature whole plants.

As used herein, transgenic plants or plant compositions refer to plants or plant compositions in which heterologous or foreign DNA is expressed or in which the expression of a gene naturally present in the plant has been altered. Such DNA will be in operative linkage with plant regulatory signals and sequences. Expression may be constitutive or may be regulatable. The DNA may be integrated into a chromosome or integrated into an episomal element, such as the chloroplast, or may remain as an episomal element. In creating transgenic plants or plant compositions, any method for introduction of such DNA known to those of skill in the art may be employed.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, DNA sequences and constructs are provided which allow for tissue-specific and/or developmentally regulated modification of gene expression, for example, during fruit maturation and ripening. Preferred sequences and constructs include transcriptional sequences which are activated at or shortly after the climacteric, so that in the early ripening of the fruit, they provide the desired level of transcription of the sequence of interest. Normally, the sequences of interest will be involved in affecting the expression of genes during ripening of the fruit or providing a property which is desirable following the growing (expansion) period of the fruit, or at or after harvesting. Desirably, the transcriptional sequences maintain their activity during the ripening or red fruit period, although the levels of their activity may also change during ripening.

As indicated above, the DNA sequences and constructs of the invention provide a regulated transcriptional sequence, which in one aspect is associated with fruit development and ripening. In one embodiment, the transcriptional sequence is one that is active upon or shortly after the onset of ripening in tomato fruit. In some DNA constructs of the invention, a sequence encoding a protein of interest is located downstream from and under the transcriptional control of the fruit-related transcriptional sequence. The protein can be a marker protein such as GUS, CAT, LUX, *etc.*, or an enzyme such as beta-fructofuranosidase, which provides for modification of the phenotype of the fruit.

The transcriptional regions may be native or homologous to the host or foreign or heterologous to the host. By foreign it is intended that the transcriptional sequence is not found in the wild-type host into which the transcriptional sequence is introduced. Of particular interest are developmentally regulated and tissue-specific (*e.g.*, fruit) transcriptional initiation regions of clone λUC82-3.3 (SEQ ID NO. 2). In tomato for example, this transcriptional region is activated upon or shortly after the onset of fruit ripening and remains active during the red fruit stage, peaking approximately midway during the ripening process. Expression of a gene coding for a protein

of interest can be developmentally controlled and made fruit-specific as well as protoxylem-specific by operatively linking the sequence or gene of interest to this transcriptional sequence from λUC82-3.3 (**SEQ ID NO. 2**).

The transcriptional sequence of the invention may, for example, be employed for varying the phenotype of the fruit. For example, the pattern of expression of genes which affect the movement and storage of fixed carbon within the plant may be modified by operatively linking these genes to heterologous promoters. For example, a transcriptional cassette may be constructed which will include in the 5'-3' direction of transcription, a transcriptional sequence, a translational initiation region, a DNA sequence encoding a protein of interest, and a transcriptional and translational termination region functional in plants. One or more introns may be also be present. The DNA sequence encoding a protein of interest may have any open reading frame encoding the peptide of interest, *e.g.,* an enzyme, or a sequence complementary to a genomic sequence, where the genomic sequence may be an open reading frame, an intron, a non-coding leader sequence, or any other sequence where the complementary sequence will inhibit transcription, messenger RNA processing, *e.g.,* splicing, or translation. The DNA sequence of interest may be synthetic, naturally derived, or combinations thereof. Depending upon the nature of the DNA sequence of interest, it may be desirable to synthesize the sequence with plant preferred codons. The plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest.

The termination region which is employed may be native with respect to the transcriptional sequence, may be native with respect to the coding DNA sequence, or may be derived from another source. Examples of termination regions from other sources include the octopine synthase and nopaline synthase termination regions derived from the Ti-plasmid of *A. tumefaciens*.

The transcription construct will normally be joined to a marker for selection in plant cells. Conveniently, the marker may be resistance to a

biocide, particularly an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, chloramphenicol, or the like. The particular marker employed will be one which will allow for selection of transformed cells as compared to cells lacking the DNA which has been introduced.

Of particular interest for the subject invention is a tissue-specific developmentally regulated transcriptional sequence (promoter) from clone λUC82-3.3 (SEQ ID NO. 2). The coding region of clone λUC82-3.3 (SEQ ID NO. 2) has homology to a bacterial histidine decarboxylase. Clone 3 λUC82-3.3 was obtained by screening on *L. esculentum* cv. UC82 genomic DNA library for clones containing sequences which hybridized to ptomUC82.1, a cDNA of tomato fruit ripening. ptomUC82.1 was identified by screening a *L. esculentum* cv. UC82 fruit cDNA library with labeled RNAs expressed either at an early green stage or at the "turning" to "pink" ripening stage of tomato fruit development; clones that hybridized strongly to the labeled RNAs were isolated and used to probe northern blots of fruit RNAs. ptomUC82.1 was identified as a cDNA corresponding to a gene expressed at low levels in early stages of fruit ripening, high levels at intermediate fruit ripening stages and decreased levels in fully ripened fruit. In addition, ptomUC82.1 was used to screen RNA from root, stem and leaf tissue. The mRNA complementary to ptomUC82.1 was not present in these tissues, nor was it detectable in green fruit.

In order to isolate the regulatory sequences associated with the developmentally expressed gene corresponding to ptomUC82.1, a tomato genomic DNA library was screened by hybridization to ptomUC82.1. A DNA fragment was selected which hybridized to the subject cDNA. The fragment is referred to herein as λUC82-3.3 (SEQ ID NO. 2). The 5′ and 3′ non-coding regions were isolated and manipulated for insertion of a foreign sequence (*e.g.*, reporter genes, enzymes, etc) to be transcribed under the regulation of the λUC82.1 promoter, thus creating expression cassettes.

The DNA constructs provided herein are introduced into plants, plant tissues, or into plant protoplasts, particularly tomato plants, plant tissues, and protoplasts, to produce transgenic plants.

Numerous methods for producing or developing transgenic plants are available to those of skill in the art. The method used is primarily a function of the species of plant. These methods include, but are not limited to, the use of vectors, such as the modified Ti plasmid system of *Agrobacterium tumefaciens*, the Ri plasmid system of *Agrobacterim rhizogenes* and the RNA virus vector, satellite tobacco mosaic virus (STMV). Other methods include direct transfer of DNA by processes such as PEG-induced DNA uptake, microinjection, electroporation, microporjectile bombardment, and direct and chemical-induced introduction of DNA (see, *e.g.,* Uchimiya *et al. J. Biotech.* *12*:1-20, 1989, for a review of such procedures).

The resulting plants may then be grown, and flowers pollinated with pollen either from the same transformed strain or different strains. The resulting hybrid, having the desired phenotypic characteristic, may then be identified. Two or more generations of homozygous transgenic plants may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited. Seeds or plant tissue then may be harvested for use in producing plants with the new phenotypic property.

In addition to SEQ ID No. 2, the invention includes sequences able to hybridize to SEQ ID No. 2, under standard high stringency conditions, (such conditions being well known to those skilled in the art of molecular plant biology), as long as those hybridizing sequences function as developmentally regulatable transcriptional sequences.

The following examples are offered by way of illustration and not by limitation.

EXPERIMENTAL

EXAMPLE 1

ISOLATION OF A DEVELOPMENTALLY REGULATED GENE

1. Construction of a cDNA library in plasmid pBR322

a. Isolation of RNA

Tomato fruit at the 3-inch intermediate stage, i.e., fruit at the "turning" to "pink" stage of development, was collected from greenhouse-grown *L. esculentum* cv. UC82 (grown from seeds obtained from Hunt-Wesson Foods,

Fullerton, CA), and frozen in liquid nitrogen. Polysomes were prepared from 10g of pulverized frozen tissue (Schroder *et al*, *Eur. J. Biochem. 67*:527-541, 1976), and RNA was extracted from the polysomes using an SDS-phenol-chloroform procedure similar to that described by Palmiter (*Biochemistry 13*:3606-3615, 1974). Poly(A)+ RNA was selected by affinity chromatography on oligo(dT)-cellulose columns using the procedure of Aviv and Leder (*Proc. Natl. Acad. Sci. USA 69*:1408-1412, 1972), except that LiCl was used instead of NaCl.

b. Preparation of cDNA and construction of cDNA library

A cDNA library was prepared by methods similar to those reported by Willa-Komaroff *et al.* (*Proc. Natl. Acad. Sci. 75*:3727-3731, 1978). USA Ten μg of poly(A)+ RNA were collected by centrifugation and resuspended in 5 μl H_2O, brought to a final concentration of 2.7 mM CH_3HgOH, and incubated at room temperature for 5 minutes (Payver and Schimke *J. Biol. Chem. 254*:7636-7642, 1979). The first strand of cDNA was synthesized by reverse transcriptase (Molecular Genetic Resources, Tampa, Florida), and mRNA was removed by treatment with NaOH. The cDNA molecules were made double-stranded by DNA polymerase I, Klenow fragment (New England BioLabs, Beverly, MA). To ensure completion of the second strand synthesis, the DNA molecules were incubated with reverse transcriptase (Molecular Genetic Resources, Tampa, 76). Following ethanol precipitation, the double-stranded molecules were digested with S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, IN). The blunt-ended molecules were then tailed with d(C) in a reaction mixture containing terminal transferase buffer (Bethesda Research Laboratories, Inc., Rockville, MD), α-^{32}P-dCTP, dCTP, and terminal transferase (Ratliff Biochemicals, Los Alamos, NM).

The d(C)-tailed DNAs were annealed to pBR322 DNA which had been digested at the *Pst*I site and tailed with d(G) (New England Nuclear, Boston, MA). The recombinant plasmid DNA molecules were used to transform LE392 *E. coli* cells which were then plated on LB-tetracycline (15 μg/ml) plates. The resultant cDNA library was stored by the procedure of Hanahan and Meselson (*Gene 10*:63-67, 1980).

2. Library screening with RNA probes

a. Preparation of ^{32}P-labeled RNA probes

Twelve grams each of 1-inch green and 3-inch intermediate *L.esculentum* cv. UC82 fruit were pulverized in the presence of liquid nitrogen, and total RNA was prepared using a phenol extraction procedure conducted at pH 9.0. Total RNAs were subjected to oligo-dT cellulose chromatography for the selection of poly(A)+ RNA essentially as described by Aviv and Leder (*Proc. Natl. Acad. Sci. USA 69*:1408-1412, 1972), except that LiCl was used instead of NaCl.

Poly(A)+ RNAs prepared from the 1-inch green and 3-inch intermediate stages of *L.esculentum* cv. UC82 tomato fruit development were fractionated on linear sucrose gradients, 5-20% sucrose, to facilitate enrichment and identification of mRNAs encoding proteins ranging in size from 30 to 60 kilodaltons.

Samples of RNA from gradient fractions were translated in an mRNA-dependent rabbit reticulocyte translation system by the method of Pelham and Jackson (*Eur. J. Biochem. 67*:247-256, 1976). The lysate and reaction conditions were as provided by New England Nuclear (Boston, MA; October 1979 Manual) to produce peptides labeled with L-(^{35}S)-methionine. Protein synthesis was assayed by determining the incorporation of TCA-precipitable label (Pelham and Jackson, *Eur. J. Biochem. 67*:247-256, 1976). The translation products were then subjected to electrophoresis on a 12.5% SDS acrylamide gel (Laemmli, *Nature 227*:680-685, 1970) and fluorography.

b. Library screening

Replica filters were prepared and the plasmids amplified (Hanahan and Meselson, *Gene 10*:63-67, 1980) using 200 μg/ml chloramphenicol. DNA from cDNA clones was denatured, neutralized, and fixed to nitrocellulose filters (Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1982).

RNAs from a gradient fraction of one-inch green fruit RNA encoding proteins with a molecular mass of approximately 30-60 kDa and from

a similar gradient fraction of three-inch intermediate fruit RNA were labeled with ^{32}P in a polynucleotide kinase reaction. These labeled fruit RNAs were then hybridized to approximately 10,000 cDNA clones (a fraction of the complete cDNA library) bound to nitrocellulose filters as described above. Of 313 clones which yielded strong hybridization signals, 36% contained insert sequences which appeared to be expressed differentially at the two different stages of development. Included in this set of cDNA clones were ptomUC82-2, ptomUC82-3, ptomUC82-6, ptomUC82-9, ptomUC82-10, and ptomUC82-22.

c. Identification of clone ptomUC82-3 as encoding a developmentally regulated sequence

Clones which yielded strong hybridization signals in the above-described hybridization experiments were used to prepare plasmid DNA which was then used to probe northern blots of total RNA prepared from the 1-inch green and 3-inch intermediate stages of *L. esculentum* cv. UC82 fruit development. Plasmid DNA was labeled with ^{32}P by nick translation.

Total RNA was prepared from 1-inch green and 3-inch intermediate developmental stages of *L. esculentum* cv. UC82 fruit as described above. The RNAs were subjected to electrophoresis on a 1.5% agarose gel prepared in 1.1 M formaldehyde, 10 mM $NaPO_4$, pH 7.4, and electrophoresed in the same buffer. The RNA was transferred to a nitrocellulose filter essentially as described by Thomas (*Proc. Natl. Acad. Sci. USA 77*:5201-5205, 1980). This filter was then cut into separate panels and hybridized with ^{32}P-labeled insert DNA from cDNA clones ptomUC82-2, ptomUC82-3, ptomUC82-6, ptomUC82-9, ptomUC82-10, and ptomUC82-22. After a 4-day exposure with an intensification screen, the autoradiographic patterns of hybridization indicated that clone ptomUC82-3 encodes a developmentally regulated, fruit-specific sequence which hybridized to a single RNA band with an apparent mobility of ~1.7 kb on a 1.5% agarose gel. Additional northern hybridization data, as well as mRNA hybridization/selection analyses, indicated that cDNA clone ptomUC82-3 corresponds to a gene which is expressed at low levels at early stages of fruit ripening, high levels at intermediate fruit ripening stages, and decreased levels

in fully ripened fruit. Sequences complementary to cDNA clone ptomUC82-3 were not detectable in RNA prepared from *L. esculentum* green fruit, leaves, stems, or roots. The insert in ptomUC82-3, which was restriction-enzyme mapped and sequenced by the dideoxynucleotide chain termination method, contained the ATG start codon and some of the coding sequence of Sequence I.D. No. 1 as well as 5' untranslated sequence.

4. Construction of a cDNA Library in λgt11 and selection of clone λtomUC82-14

a. Total RNA extraction and poly(A) mRNA isolation

Tomato fruit at the 3-inch intermediate stage was collected from greenhouse-grown *L. esculentum* cv. UC82 as described above. Procedures used for total RNA extraction and poly(A) mRNA isolation were essentially as described in section 2.a. (*supra*).

b. cDNA preparation and library construction in λgt11

cDNA was prepared using reverse transcriptase, made double-stranded with DNA polymerase I, and made blunt-ended by treatment with S1 nuclease. Oligonucleotide adapter molecules as described by Wood *et al.* (*Nature 312*:330-337, 1984) were used to join the blunt-ended, double-stranded DNA to the λgt11 vector DNA. Ligation of the blunt-ended, double-stranded DNA product from the cDNA reactions to the adapter molecules was accomplished by incubating the DNA with a 50-fold excess of adapters.

The DNA products of this ligation reaction were phosphorylated by incubating them in a polynucleotide kinase reaction. Phosphorylated DNA with an apparent size greater than 1 kilobase pair was collected following fractionation on a Sepharose CL-4B column. The phosphorylated DNA molecules were then ligated to λgt11 arms and packaged using a Gigapack^{R} lambda packaging extract obtained from Stratagene (La Jolla, CA). The resultant cDNA library contained ~2 x 10^5 p.f.u.; the library was amplified prior to screening.

c. Screening of the library and identification of λtomUC82-14

Approximately 2 x 10^4 p.f.u. of the amplified library were plated on *E. coli* Y1088 cells and screened with ^{32}P-labeled insert DNA derived from

ptomUC82-3. To prepare this probe, ptomUC82-3 plasmid DNA was digested with *Pst*I, the ~800 bp insert fragment was fractionated on an agarose gel and purified, and the fragment was labeled with ^{32}P in a nick translation reaction. Plaques hybridizing to this probe were identified and plaque-purified. Following plaque purification, the insert sizes of these recombinant bacteriophage were determined, and the clone containing the largest insert was selected for restriction enzyme analysis and sequencing by the dideoxynucleotide method. This clone was named λtomUC82-14, and the sequence of the insert DNA contained within this clone extends from nucleotides 7 through 1576 of Sequence I.D. No. 1.

EXAMPLE 2

ISOLATION OF TOMATO HDC PROMOTER

1. Construction and screening of genomic library.

A genomic library was constructed in λ FIX™II (Stratagene, La Jolla, CA) using DNA isolated from seedling tissue of *L. esculentum* cv. UC82. The genomic library was screened with a ^{32}P-labeled probe prepared from the 800 bp insert purified from cDNA ptomUC82-3 following digestion with *Pst*I. The hybridizations were conducted overnight at 42°C in 50% formamide, 5X SSPE, 5X Denhardt's solution, 0.1% SDS, and 200 μg denatured salmon sperm DNA. The screening resulted in the identification and plaque-purification of 13 clones which hybridized to the insert.

2. Isolation of HDC Promoters

One of the clones isolated from the genomic DNA library, λUC82-3.3, containing nucleic acids 1-4032 of Sequence I.D. No. 2., was shown by restriction enzyme mapping to contain putative regulatory regions upstream of the translation start site. A 3.7 kb *Sst*I-*Bgl*II fragment from the 5′ end of this clone was subcloned. Sequence analysis of the insert of this subclone revealed that it contains six exons that have 95-100% identity with comparable positions of ptomUC82-3 cDNA, and appears to include a promoter region. A fragment containing the 347 bp upstream from the *Sst*I restriction site near the 5′ end of the λUC82-3.3 insert was subcloned and sequenced.

The results of a sequence similarity search through the GenBank database release 67.0 and EMBL database release 26.0 (Devereaux *et al.*, *Nucl. Acids Res. 12*:387-395, 1984) indicate a 60% similarity between the amino acid sequences predicted from cDNA clone ptomUC82-3 and the *Morganella morganii* bacterial histidine decarboxylase gene. Thus, the *L. esculentum* gene identified by hybridization to the cDNA clone ptomUC82-3 probe is considered to be a histidine decarboxylase-like (HDC) gene.

The promoter-containing region of λUC82-3.3, nucleotides 1-888 of **SEQ ID No. 2**, is herein referred to as the HDC promoter.

EXAMPLE 3

HDC-PROMOTER/TOMATO FRUIT INVERTASE CONSTRUCTS

1. HDC/3-L1.1

Construct HDC/3-L1.1 contains 538 bp of the HDC promoter region from λUC82-3.3 (nucleotides 349 to 886 of Sequence I.D. No. 2) fused to the coding sequence of *L. esculentum* cv. UC82 invertase cDNA, which is fused at the 3′ end to the NOS (nopaline synthase) terminator, as shown in Figure 2.

pTOM3-L1 was digested with *Xho*I, made blunt-ended with T4 DNA polymerase, then digested with *Not*I to yield a 2202-bp fragment containing 3 nucleotides from the vector polylinker (AGC) plus the complete *L. esculentum* cv. UC82 invertase cDNA coding sequence.

The above fragment prepared from pTOM3-L1 and the 538 bp fragment of the HDC promoter (nucleotides 349 to 886 of Sequence I.D. No. 2) were purified and ligated with *Not*I- and *Sst*I-digested pGEM-11Zf(-) (Promega Corporation, Madison, WI). The resulting plasmid was called -540/3-L1.

The NOS terminator is contained in plasmid pBI101 (Clontech, Palo Alto, CA). Plasmid pBI101 was digested with *Sst*I and *Hind*III, made blunt-ended with T4 DNA polymerase, yielding an ~10-kb vector fragment. The purified vector fragment was ligated to the DNA insert of -540/3-L1 which had been prepared by digestion with *Not*I and made blunt-ended with T4 DNA polymerase, to produce construct HDC/3L-1.1.

2. HDC/3-L1.2

Construct HDC/3-L1.2 contains 886 bp of the HDC promoter region from λUC82-3.3 (nucleotides 1 to 886 of Sequence I.D. No. 2) fused to the *L. esculentum* cv. UC82 invertase cDNA, which is fused at the 3′ end to the NOS (nopaline synthase) terminator, as shown in Figure 2.

3. HDC/3-L1.3

Construct HDC/3-L1.3 contains 690 bp of the HDC promoter region from λUC82-3.3 (nucleotides 1 to 690 of Sequence I.D. No. 2) fused to the *L. esculentum* cv. UC82 invertase cDNA which is fused at the 3′ end to the NOS (nopaline synthase) terminator, as shown in Figure 2.

EXAMPLE 4

HDC-PROMOTER/GUS CONSTRUCTS

1. HDC/GUS.1

Construct HDC/GUS.1 contains the promoter fragment from λUC82-3.3 which extends from 794 to 3 bp upstream of the ATG start codon (nucleotides 94 to 886 in Sequence I.D. No. 2) fused to the *E. coli* β-glucuronidase (GUS) gene as shown in Figure 3.

Plasmid pUC82-3.3NH was digested with *Dde*I, the ends of the resultant fragment were filled in with DNA polymerase I, Klenow fragment, and the 792 bp fragment was isolated and purified. Plasmid pUC82-3.3NH was constructed by inserting the 3.4-kb *Hind*III fragment, which extends from the *Not*I site in the vector polylinker to the first *Hind*III site from the 5′ end of the λUC82-3.3 insert, into the *Not*I and *Hind*III sites of pGEM-11Zf(-) (Promega Corporation, Madison, WI) to produce pUC82-3.3NH.

Plasmid pBI101.3/pUC was made by inserting the 2200 bp *Eco*RI-*Hind*III fragment of pBI101.3 (Clontech, Palo Alto, CA) into *Eco*RI and *Hind*III-digested pUC119 (Vieira and Messing, in *Methods in Enzymology*, R. Wu and L. Grossman, eds. Vol. 153, pp. 3-11, Academic Press, New York, 1987). The 792 bp fragment was ligated to pBI101.3/pUC which had been digested with *Hin*dIII and *Bam*HI, and the resulting plasmid was called -790/GUS.

The 3 kb *Eco*RI-*Hin*dIII fragment containing the HDC promoter-GUS fusion was isolated from -790/GUS and ligated to *Eco*RI- and *Hin*dIII-digested pBIN19 (Clontech, Palo Alto, CA) to produce HDC/GUS.1.

2. HDC/GUS.2

Construct HDC/GUS.2 contains 690 bp of the HDC promoter region from λUC82-3.3 (nucleotides 1 to 690 of Sequence I.D. No. 2) fused to the *E. coli* GUS gene, as shown in Figure 3.

Plasmid pUC82-3.3NH was digested with *Xba*I and *Ssp*I, and the 710-bp fragment was isolated on a 1% agarose gel and purified. The fragment was ligated to gel-purified *Xba*I- and *Sma*I-digested pBI101.3/pUC to create -690/GUS.

The 2.9-kb *Eco*RI-*Hin*dIII fragment containing the HDC promoter-GUS fusion was isolated from -690/GUS and ligated to *Eco*RI- and *Hin*dIII-digested pBIN19 (Clontech, Palo Alto, CA) to produce HDC/GUS.2.

EXAMPLE 5

TRANSFORMATION OF TOMATO PLANTS WITH HDC PROMOTER CONSTRUCTS

1. Transformation of *L. esculentum* seedlings

The transformation of seedlings of *L. esculentum* cv. UC82 (grown from seeds obtained from Ferry Morse Seed Co., Modesto, CA) was done essentially according to the protocol of Fillatti *et al.* (*Bio/Technology* 5:726-730, 1987). Plasmids were inserted into *Agrobacterium tumefaciens* strain LBA4404 (Clontech, Palo Alto, CA; see also Ooms *et al.*, *Plasmid* 7:15-19, 1982) through triparental mating for transfer into *L. esculentum* tissue.

The cultures were incubated at 27°C with 16 hours of light per day under 4,000 lux of light intensity. When kanamycin-resistant shoots reached a height of one inch, they were rooted on rooting medium. The transgenic shoots were then grown into fruit-bearing transgenic tomato plants.

2. Assays for Recombinant Gene Expression

Since the HDC promoter sequences are developmentally regulated and fruit-specific, tomato fruit tissues are assayed for invertase or GUS expression at various stages of fruit development.

Invertase activity is assayed at 30°C on 50mM sucrose in 13.6M citric acid and 26.4 mM disodium phosphate (pH 4.8). The reaction is stopped with the alkaline copper reagent of Somogyi (*J. Biol. Chem. 160*:61-68, 1945). The liberated reducing sugars are measured according to Nelson (*J. Biol. Chem. 153*:375-380, 1944). Substrate specificity is determined by reacting samples (for example, ~4 μg of protein obtained following Concanavlin A-Sepharose column chromatography) with 90 mg/ml of substrate (sucrose or raffinose), in 40 mM citric acid-$NaHPO_4$ buffer, pH 4.8, at 30°C for 30 minutes. The products of these reactions are then analyzed by thin layer paper chromatography using isobutanol:pyridine:H_2O:acetic acid (12:6:4:1) as the solvent for ascending chromatography (Gordon *et al., J. Chromatog. 8*:44-59, 1962). The positions of the carbohydrates are detected with alkaline silver nitrate (Chaplin, "Monosaccharides", in *Carbohydrate Analysis, A Practical Approach*, Chaplin and Kennedy, eds; IRL Press, Washington, DC, pp. 1-36, 1986).

GUS activity was determined according to the protocols provided by Jefferson (*Plant Mol. Biol. Rep. 5*:387-405, 1987). Histochemical analysis of 3-inch intermediate ("turning" to "pink") *L. esculentum* cv. UC82 transgenic fruit indicated that GUS expression (under the control of the HDC promoter) was localized to protoxylem tissue. This result was observed in plants transformed with either HDC/GUS.1 or HDC/GUS.2.

The above results demonstrate the ability to identify inducible regulatory sequences in a plant genome, isolate the sequences and manipulate them. In this way, the production of transcription cassettes and expression cassettes can be produced which allow for differentiated cell production of the desired product. Thus, the phenotype of a particular plant part may be modified, without requiring that the regulated product be produced in all tissues, which may result in various adverse effects on the growth, health, and production capabilities of the plant. Particularly, tissue specific (*e.g.*, fruit specific) transcription initiation capability is provided for modifying the phenotypic properties of a variety of fruits to enhance properties of interest such as processing, organoleptic properties, storage, yield, or the like.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

SUMMARY OF SEQUENCES

SEQUENCE ID NO. 1: a cDNA clone of pTOMUC82.1 from *Lycopersicon esculentum*

SEQUENCE ID NO. 2: a genomic clone of λ UC82-3.3 from *Lycopersicon esculentum*

SEQUENCE LISTINGS

(1) GENERAL INFORMATION:

(i) APPLICANT: Fitzmaurice Ph.D., Leona C.
Mirkov Ph.D., T. Erik
Elliot Ph.D., Kathryn
Holtz, Greg
Dickinson, Craig

(ii) TITLE OF INVENTION: Tissue-Specific Developmentally Regulated Transcriptional Sequences and Uses Thereof

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: McCubbrey, Bartels, Meyer, & Ward

(B) STREET: One Post St.

(C) CITY: San Francisco

(D) STATE: CA

(E) COUNTRY: USA

(F) ZIP: 94104-5231

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/635,820

(B) FILING DATE: 02-JAN-1991

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/352,658

(B) FILING DATE: 18-MAY-1989

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/343,466

(B) FILING DATE: 26-APR-1989

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/197,122

(B) FILING DATE: 20-MAY-1988

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Meyer Esq., Virginia H.

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(C) REFERENCE/DOCKET NUMBER: 51651M

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415) 391-6665

(B) TELEFAX: (415) 391-6663

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1576 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Lycopersicon esculentum

(vii) IMMEDIATE SOURCE:

(B) CLONE: pTOMUC82.1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGAAATTC AAAAGGAGTT TGATTTAACG GTAGTTCCAA CAGAAGGTGA AATTGATGCA 60
CCATTATCGC CAAGGAAGAA TTTATGTCTC AGTGTGGTGG AATCCGATAT AAAAAATGAA 120

ACGTCTTTTC AAAAACTTGA CATGATTTTG ACTCAATATT TAGAGACATT GTCAAAACGG 180

AAGAAGTATC ATATAGGTTA TCCAACAAAC ATGCATTATG AGCATCATGC CACTTTAGCC 240

CCACTTTTGC AATTTCATTT GAACAATTTT GGAGACCCCT TTGCTCAGCA CCCTACAGAT 300

TTTCATTCAA AAGATTTTGA AGTGGCTGTA TTAGATTGGT TTGCACAACT CTGGGAAATA 360

GAGAAAGATG AATATTGGGG ATACATTACT AGTGGTGGCA CTGAGGGCAA TCTCCATGGC 420

CTTTTGGTTG GAAGAGAGCT ACTTCCAAGT GGGATATTAT ATGCATCAAA AGATTCACAT 480

TACTCAATTT TCAAAGCAGC AAGAATGTAT CGAATGGAGC TACAAACTAT CAACACTTTA 540

GTTAATGGGG AAAATGATTA TGAAGATTTA CAATCAAAGT TACTTGTCAA CAAGAACAAA 600

CCAGCTATCA TCAATATCAA TATTGGAGCT ATTGATGACC TCGATTTCGT CATACAAACA 660

CTTGAAAATT GTGGTTATTC AAATGACAAT TATTATATCC ATTGCGATGC AGCATTATGT 720

GGGCTAATTC TCCCATTTAT CAAACATGCA AAAAAAATTA CCTTCAAGAA GCCAATTGGT 780

AGTATTTCAA TTTCAGGGCA CAAATTCTTG GGATGTCCAA TGCCTTGTGG CATTCAGATA 840

ACAAGGAAAA CTTATGTTAG TACCCACTCA AAAATTGAGT ATATTAATTC CACAGATGCT 900

ACAATTTCTG GTAGTCGAAA TGGATTTACA CCAATATTCT TATGGTACTG TTTAAGCAAG 960

AAAGGACATG CTAGATTGCA ACAAGATTCC ATAACATGCA TTGAAAATGC TCGGTATTTG 1020

AAAGATCGAC TTCTTGAAGC AGGAATTAGT GTTATGCTGA ATGAGTTTAG TATTACTGTT 1080

ATTTTTGAAC GATCTTGTGA CCATAAATTC ATTCATCGTT GGAACTTGTG TTACTTAAGA 1140

GGCATGGCAC ATGTTGTGGT TATGCCAGGT ATTACAAGAG AAACTATAGA CAGTTTCTTC 1200

AAAGATCTAA TGCAAGAGAG GAAGAGGTGG TTTCAGGATG GAAAAAACTC AGCCTCCTTG 1260

TCTAGCAGAT GAGTTTGGAT CTCAAAATTG TATGTGCTCC CATAACAAGA TGCATAACTA 1320

AACTCCTTGG AACCATGACT TGAAATGGTC ATGATTATCA AGTATGTTTT TGATGCAAGA 1380

GTGACTCAAT AAAATTTATG ATCTAAATCG ATCTATAGTT TTCTAATAAA TTTATATGTA 1440

TACTTTCTTT GTTGTGCTTT TACACGAATG TTACTCAATA AAATTTGTAA TATAGAGTCA 1500

TTTAGAGTTT TCAAATCAAT TTTTATGTAT ACGTTGTTTA CAAATTTTGT AATTTAACCC 1560

TTGACCGTAA GACATG 1576

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4032 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Lycopersicon esculentum

(vii) IMMEDIATE SOURCE:

(B) CLONE: lambda UC82-3.3

(ix) FEATURE:

(A) NAME/KEY: prim_transcript

(B) LOCATION: 889

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCAAATTT AGTTTTGACA TCTTCTTCAC ATTTCAAGCA TTAAAACCAA TTAACACTGT 60

TTTATTATTA TTATTATATT AATTTAAATT TTCTGAGTTT AATTTTATTA TTCTAACATT 120

ATTTTATATA CTTTTCATTG AAAAATTGCA TTGTTTATAT TCTTACTTCA TAATGTACGT 180

ATATAACATT CTTTGCAGAC TTCATTTATG AAATTACACT ATAGAATAAT AATTTGATTT 240

ATATGTACTT CCTTCCTTTC AAATTGATTA AATTGTTAAG GTGTTTCACA CATTTAAAAT 300

AAATTAAGTC ACATATTAAG CATAACTTTA AATTTTTACA AAAATAAGAG CTCTCTATAA 360

AGTTTGACTT TAAGTCTCCA AATTTGTTAA TACAGACCTG AAAGAGTGTA GGAGCTAACA 420

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ATACACCAAA ATTTTACATC CTTTATCATA GCAAAATTTA TAGAAAATAA AAATAAATTT 540

GTAACTAATG TTTTTTTTTT CAAACACTGT AAAACACGAA AAAAATTGCT AATGTGTAAG 600

AAAACATGTG TAATATAAAA CAAATATAAA AGAGTCCACG TGCATCGCAT GAGTACCTAT 660

ATTAATTTTA GCTTGAAAAT AAAAATTAAT ATTTTTTTAT TTCAAACACT ACCAATTATT 720

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AATCAATGAC AAATTTGTAT TGCCACACAA TAATGACCAC AATCCTTCTA TGCAAGAGCT 1980

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AACTCGAATT GATGTCTTAC CCTCTTGGTG CGATAGAACT TGAATCTACC GTCTTACCCT 2940

CAAATATTTT TCATAATGAA TGACATAAAT GAGTCTTTTT TAAACAAATT TGATAACATA 3000

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CTTACATCTT ATAACATCAC TTTTGGTAAA TTAGTATATA TGTGTTTATA GGAACAACCT 3360

TCAAAGGAGC TATTGATGAC CTCGATTTCG TCATACAAAC ACTTGAAAAT TGTGGTTATT 3420

CAAATGACAA TTATTATATC CATTGCGATG CAGCATTATG TGGGCTAATT CTCCCATTTA 3480

TCAAACATGT AAGCTTATTT TTATTCAATT TTCCTTCAAC GCTCGATCGA AGTTACAATG 3540

ACATAGTTTC TTTCTATGGT ATTTGACAAT AGGCAAAAAA AATTACCTTC AAGAAACCAA 3600

TTGGAAGTAT TTCAATTTCA GGGCACAAAT TCTTGGGATG TCCAATGTCT TGTGGCGTTC 3660

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GCAAGAAAGG ACATGCTAGA TTGCAACAAG ATTCCATAAC ATGCATTGAA AATGCTCGGT 3840

ATTTGAAAGA TCGACTTCTT GAAGCAGGAA TTAGTGTTAT GCTGAATGAT TTTAGTATTA 3900

CTGTTGTTTT TGAACGACCT TGTGACCATA AATTCATTCG TCGTTGGAAC TTGTGTTGCT 3960

TAAGAGGCAT GGCACATGTT GTAATTATGC CAGGTATTAC AAGAGAAACT ATAGATAGTT 4020

TCTTCAAAGA TC 4032

CLAIMS:

1. DNA comprising **SEQ ID NO. 2** (λUC82-3.3).

2. DNA able to hybridize under standard high stringency conditions with the DNA of Claim 1, wherein said hybridizing DNA functions as a developmentally regulatable transcriptional DNA sequence.

3. A DNA construct comprising in the direction of transcription, (a) a transcriptional region from **SEQ ID NO. 2**, or a sequence able to hybridize thereto under standard high stringency conditions wherein said hybridizing DNA functions as a developmentally regulatable transcriptional DNA sequence; operatively linked to (b) a DNA sequence of interest, wherein said DNA sequence of interest is other than the wild-type sequence normally associated with said transcriptional region, and wherein said DNA sequence is under the transcriptional regulation of said region or said hybridizing sequence; and (c) a transcriptional termination region.

4. A DNA construct according to Claim 3, wherein said DNA sequence of interest encodes protein(s) which directly or indirectly gives rise to a phenotypic trait wherein said phenotypic trait is selected from the group consisting of tolerance or resistance to: herbicide, fungus, virus, bacterium, insect, nematode or arachnid; production of secondary metabolites, male or female sterility, and production of an enzyme or reporter compound.

5. A DNA construct according to Claim 3, wherein said DNA sequence of interest encodes reporter protein(s) selected from the group consisting of chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), nopaline synthase (NOS), octopine synthase (OCS), β-1,3-glucuronidase (GUS), acetohydroxyacid synthase (AHAS), β-galactosidase (βGAL), and luciferase (LUX).

6. A DNA construct for integration into a plant genome comprising at least the right T-DNA border joined to a DNA construct according to Claim 3.

7. A DNA vector comprising a broad spectrum prokaryotic replication system and a DNA construct according to Claim 3.

8. A plant transformed with a DNA construct according to Claim 3.

9. A tomato plant transformed with a DNA construct according to Claim 3.

10. A method for modifying the phenotype of fruit in a plant, said method comprising: transforming a suitable plant host cell with a DNA construct according to Claim 3, under genomic integration conditions, whereby said DNA construct becomes integrated into the genome of said plant host cell; regenerating a plant from said transformed plant host cell; and growing said plant to produce fruit of the modified phenotype.

11. A method for modifying the phenotype of a tomato fruit, said method comprising: transforming a suitable tomato plant host cell with a DNA construct, under genomic integration conditions, wherein said DNA construct comprises in the direction of transcription, (a) a tomato HDC-like transcriptional region from **SEQ ID NO. 2**, or a sequence able to hybridize thereto under standard high stringency conditions wherein said hybridizing DNA functions as a developmentally regulatable transcriptional DNA sequence; operatively linked to (b) a DNA sequence of interest, wherein said DNA sequence of interest is other than the wild-type sequence normally associated with said transcriptional region, and wherein said DNA sequence is under the transcriptional regulation of said region or said hybridizing sequence, and further wherein said DNA sequence is capable of modifying the phenotype of fruit cells upon transcription; and (c) a transcriptional termination region; whereby said DNA construct becomes integrated into the genome of said plant host cell; regenerating a plant from said transformed plant host cell; and growing said plant to produce fruit of the modified phenotype.

12. DNA comprising **SEQ ID NO. 1** (pTOMUC82.1).

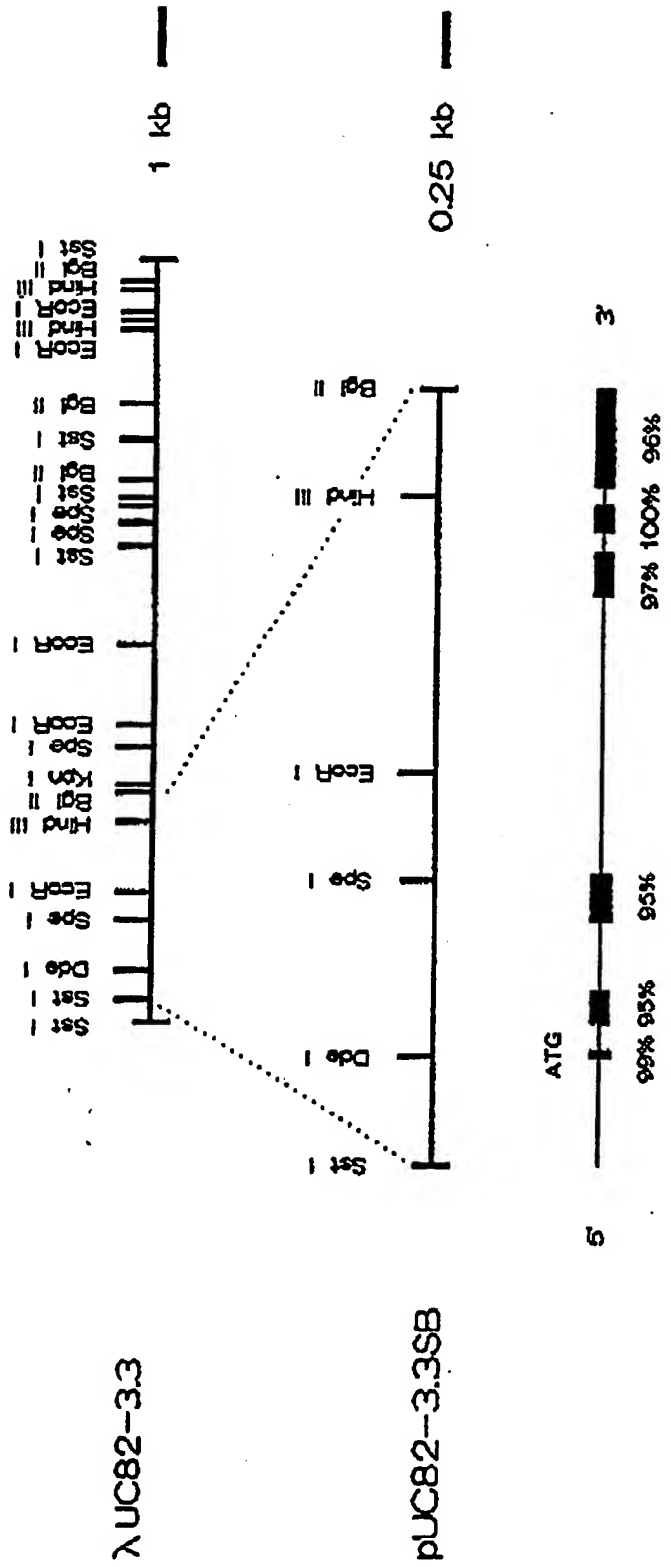

Figure 1